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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
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Method of treating or preventing estrogen-suppressed tumours

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METHOD OF TREATING OR PREVENTING ESTROGEN-SUPPRESSED TUMOURS

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TECHNICAL FIELD OF THE INVENTION

(51)

The present invention relates to a method for treating or preventing estrogen-suppressed tumours in a mammal by administering an effective amount of a special estrogenic component to said mammal. The present method is particularly suitable for treating or preventing colorectal and prostate cancer.

BACKGROUND OF THE INVENTION

Colorectal carcinoma is a malignant neoplastic disease. There is a high incidence of colorectal carcinoma in the Western world. Tumours of this type often metastasize through lymphatic and vascular channels. Many patients with colorectal carcinoma eventually die from this disease. To date, systemic therapies and chemotherapies have been developed for the treatment of colorectal cancer. However, no therapies have exhibited sufficient anti-tumour activity to prolong the survival of colorectal carcinoma patients with metastatic disease with any degree of reliability. As a result, a need still exists to develop methods for the successful treatment or prevention of colorectal carcinoma.

In an article by Al-Azzawi et al. ("Estrogen and colon cancer: current issues", *Climacteric* 2002;5:3-14) current epidemiological data on the incidence and mortality of colon cancer in post-menopausal women using hormone replacement therapy is reviewed. Hormone replacement therapy (HRT) comprises the administration of estrogen to prevent or treat symptoms resulting from estrogen deficiency (hypoestrogenism). The authors conclude that estrogen use confers overall protection, with a reduction in the incidence of colon adenoma and carcinoma of about 30%. It is said that estrogen use reduces the colon cancer-related mortality.

US 6,291,456 (Signal Pharmaceuticals Inc.) relates to compounds that modulate gene expression through the estrogen receptor as well as to methods for treating a large number of estrogen-related conditions, including colon cancer. The compounds according to this US-

patent include both estrogen antagonists and agonists. It is observed in the patent that those compounds that are estrogen antagonists are useful as antiestrogens in, for example, breast and ovarian tissue and thus are useful in the treatment and prevention of breast and ovarian cancer. Those compounds that are estrogen agonists are recommended for other therapeutic and prophylactic uses.

As will be evident from the above publications, it has been suggested in the prior art that the administration of estrogens to postmenopausal females may help to reduce the risk of colon cancer. However, this observation does not automatically warrant the conclusion that it would be advisable to treat or prevent estrogen-suppressed cancers, such as colon cancer, by administering estrogen. Indeed, it is well known that estrogens increase the risk of "estrogen-stimulated cancers", e.g. endometrial cancer in females (Cushing et al., 1998. Obstet. Gynecol.91, 35-39; Tavani et al., 1999. Drugs Aging, 14, 347-357) and breast cancer in both females and males (Tavani et al., 1999. Drugs Aging, 14, 347-357; Pike et al., 2000. Steroids, 65, 659-664, Heinig et al. 2002, European Journal of Obstetrics & Gynecology, 102, 67-73), by inducing an estrogen receptor mediated increase in the frequency of cell division (proliferation) within these tissues. Cell division is essential in the complex process of genesis of human cancer since it *per se* increases the risk of genetic error, particularly genetic errors such as inactivation of tumour suppressor genes.

Since the incidence of the aforementioned estrogen-stimulated cancers in industrialised countries is very high, the administration of estrogens in the treatment or prevention of e.g. colon cancer is associated with very significant drawbacks. Thus, there is a requirement for an estrogen that could be employed in the treatment of estrogen-suppressed tumours without enhancing the risk of estrogen-stimulated tumours.

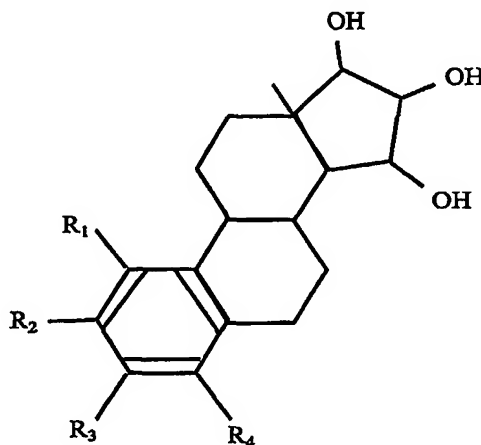
Prostate cancer is the second leading cause of cancer mortality in men in the USA. For the past six decades, hormonal therapy has been an important treatment of advanced prostate cancer. One such method employs diethylstilbestrol (DES) to suppress endogenous androgen production. DES is a substance that is known to exhibit estrogenic activity. However, the use of DES is marred by significant cardiovascular toxicity. Strategies to reduce thromboembolic events, such as dose reduction or the use of warfarin sodium were unsuccessful (Malkwicz et al., "The role of diethylstilbestrol in the treatment of prostate cancer", Urology 2001 Aug;58(2 Suppl 1):108-13). In addition, the application of DES is believed to enhance the risk of breast cancer.

Consequently, there is a need for an estrogenic substance that may be used in the treatment or prevention of estrogen-suppressed tumours, such as colorectal tumours or

prostate tumours, and which does not impose a risk factor for the development of estrogen-stimulated cancers or display significant cardiovascular toxicity.

5 SUMMARY OF THE INVENTION

The inventors have unexpectedly found that the aforementioned requirements are met by estrogenic substances that are represented by the following formula



- 10 in which formula R₁, R₂, R₃, R₄ independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms.

A known representative of this group of estrogenic substances is 1,3,5 (10)-estratrien-3, 15 α ,16 α ,17 β -tetrol, also known by the names of estetrol, oestetrol and 15 α -hydroxyestriol. Estetrol is an estrogen that is produced by the fetal liver during human pregnancy.

- 15 Unconjugated estetrol levels in maternal plasma peak at about 1.2 ng/ml at term pregnancy and are about 12 times higher in fetal than in maternal plasma (Tulchinsky et al., 1975. J. Clin. Endocrinol. Metab., 40, 560-567).

- It is very surprising that the present estrogenic substances do not enhance the risk of estrogen-stimulated tumours as the skilled person would expect estrogenic substances to
20 enhance the formation and growth of such tumours. Since the present estrogenic substances do not appear to exhibit estrogen antagonistic properties, this finding is truly unexpected.

The present estrogenic substances were found to exhibit a relatively high affinity for the ER α receptor, or conversely a relatively low affinity for the ER β receptor. It is believed that this receptor specificity is somehow associated with the observation that the present substances, unlike commonly used estrogens, do not stimulate proliferation in estrogen-stimulated tissues. However, the mechanisms that govern the ER signalling pathways that are responsible for this phenomenon are as yet poorly understood, despite the considerable scientific effort that is ongoing in this area.

It is known that most estrogens bind to both ERs which, in the presence of tissue-specific co-activators and/or co-repressors, bind to an estrogen response element in the regulatory region of genes or to other transcription factors. Given the complexity of ER signalling, along with the tissue-specific expression of ER α and ER β and its co-factors, it is now recognised that ER ligands can act as estrogen agonists or even as estrogen antagonists in a tissue-specific manner.

It is also now known that estrogen modulates cellular pharmacology through gene expression, and that the estrogen effect is mediated by the estrogen receptors. The effect of the estrogen receptor on gene regulation can be mediated by a direct binding of ER to the estrogen response element, binding of ER to other transcription factors such as NF- κ B, C/EBP β and through non-genomic effects involving ion channel receptors. Progress over the last few years has shown that ER associates with co-activators (e.g., SRC-1, CBP and SRA) and co-repressors (e.g., SMRT and N-CoR), which also modulate the transcriptional activity of ER in a tissue-specific and ligand-specific manner. In addition, evidence now suggests that the majority of estrogen-regulated genes do not have a classical estrogen response element. In such cases, ER interacts with the transcription factors critical for regulation of these genes. Transcription factors known to be modulated in their activity by ER include, for example, AP-1, NF- κ B, C/EBP and Sp-1.

Given the complexity of ER signalling, as well as the various types of tissue that express ER and its co-factors, it is commonly believed that ER ligands can no longer simply be classified as either pure antagonists or agonists. This view is supported by the findings of Paech et al. (Science 277, 1508-1510, 1997) who have reported that 17 β -estradiol activates an AP-1 site in the presence of ER α , but inhibits the same site in the presence of ER β . In contrast, the ER ligands raloxifene (Eli Lilly & Co.) and tamoxifen and ICI-182,780 (Zeneca Pharmaceuticals) stimulate the AP-1 site through ER β , but inhibit this site in the presence of ER α .

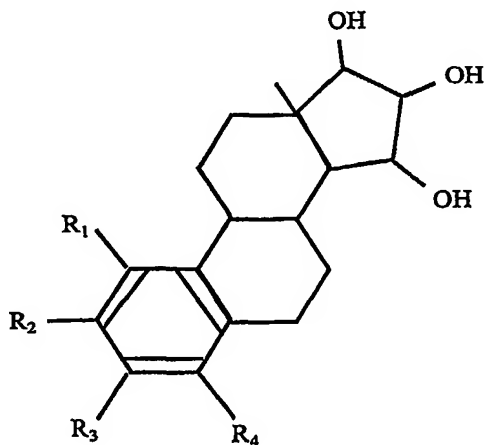
ER α and ER β are known to have both overlapping and different tissue distributions, as analysed predominantly by RT-PCR or in-situ hybridisation. Very often tissues express both ER α and ER β , but the receptors are localised in different cell types.

In summary, although the mechanisms by which the present estrogenic component exerts its favourable effect are as yet unknown, it is evident that said estrogenic component is different from estrogenic substances, such as 17 β -estradiol and ethinyl estradiol, in that it exhibits a relatively high affinity for the ER α receptor in comparison to the ER β receptor. It will also be clear from the above that this specificity may well be responsible for the efficacy of the present estrogenic component in the treatment or prevention of estrogen-suppressed tumours without the disadvantage of enhancing the risk of estrogen-stimulated tumours in both females and males.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the present invention relates to a method of treating or preventing estrogen-suppressed tumours in a mammal, said method comprising the administration of a therapeutically effective amount of an estrogenic component to said mammal, wherein the estrogenic component is selected from the group consisting of:

substances represented by the following formula



in which formula R₁, R₂, R₃, R₄ independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms;

precursors capable of liberating a substance according to the aforementioned formula when used in the present method; and mixtures of one or more of the aforementioned substances and/or precursors.

As used herein the term "tumour" refers to a new growth of tissue in which the multiplication of cells is uncontrolled and progressive. The term tumour encompasses both malignant and benign tumours.

The term "estrogen-suppressed tumour" refers to a tumour whose formation and growth is suppressed by the administration of estrogens and is not restricted to tumours whose formation and growth are directly affected by estrogens. For instance, estrogen-suppressed tumours also encompasses tumours whose formation and growth are stimulated by androgens and whose formation and growth may be suppressed by administration of estrogen in an amount effective to inhibit endogenous production of androgens. Examples of such androgen-stimulated tumours that are to be regarded as estrogen-suppressed tumours include prostate tumours and prostate hypertrophy.

The term "estrogen-stimulated tumour" refers to a tumour whose formation and growth is stimulated by (endogenous or exogenous) estrogens, other than the estrogenic components according to the present invention, especially estrogens selected from the group consisting of 17β -estradiol, conjugated equine estrogens, ethinyl estradiol, as well as precursors and metabolites thereof.

The term "cancer" refers to cells that have undergone a malignant transformation that makes them pathological to the host organism.

The present estrogen substances are distinct from both the biogenic and synthetic estrogens that are commonly applied in pharmaceutical formulations in that the 5 membered ring in the steroid skeleton comprises 3 hydroxyl substituents rather than 0-2. In a particularly preferred embodiment at least one of R_1 , R_2 , R_3 and R_4 represents a hydroxyl group, meaning that the estrogen substance contains at least 4 hydroxyl groups. Preferably, the estrogenic component applied as the active component in the present composition is a so called biogenic estrogen, i.e. an estrogen that occurs naturally in the human body, a precursor of a biogenic estrogen or a mixture thereof. Because biogenic estrogens are naturally present in the fetal and female body, side-effects are not expected to occur, particularly not if the serum levels resulting from the exogenous administration of such estrogens do not substantially exceed naturally occurring concentrations.

In a preferred embodiment of the present invention the estrogenic substance contains 4 hydroxyl groups. In another preferred embodiment, no more than 3 of R_1 , R_2 , R_3 , R_4 are

hydrogen atoms. Also, in the aforementioned formula, R_1 preferably represents a hydrogen atom. In said formula preferably at least 2, more preferably at least 3 of the groups R_1 , R_2 , R_3 and R_4 represent a hydrogen atom.

The estrogenic substances according to the formula encompass various enantiomers since the carbon atoms that carry hydroxyl-substituents are chirally active. In one preferred embodiment, the present estrogenic substance is 15α -hydroxy substituted. In another preferred embodiment the substance is 16α -hydroxy substituted. In yet another preferred embodiment, the substance is 17β -hydroxy substituted. Most preferably the estrogenic substances are $15\alpha, 16\alpha, 17\beta$ -trihydroxy substituted.

In a preferred embodiment of the present invention R_3 represents a hydroxyl group or an alkoxy group. In another preferred embodiment the groups R_1 , R_2 and R_4 represent hydrogen atoms, in which case the substance is 1,3,5 (10)-estatrien-3, 15,16,17-tetrol. A preferred isomer of the latter substance is 1,3,5 (10)-estatrien-3, $15\alpha, 16\alpha, 17\beta$ -tetrol (estetrol).

The invention also encompasses the use of precursors of the estrogen substances that constitute the active component in the present method. These precursors are capable of liberating the aforementioned estrogen substances when used in the present method, e.g. as a result of metabolic conversion. These precursors are preferably selected from the group of derivatives of the present estrogen substances, wherein the hydrogen atom of at least one of the hydroxyl groups has been substituted by an acyl radical of a hydrocarbon carboxylic, sulfonic acid or sulfamic acid of 1-25 carbon atoms; tetrahydrofuranyl; tetrahydropyranyl; or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue. Typical examples of precursors which can suitably be used in accordance with the invention are esters that can be obtained by reacting the hydroxyl groups of the estrogen substances with substances that contain one or more carboxy ($M^+ \text{OOC}-$) groups, wherein M^+ represents a hydrogen or (alkali)metal cation. Hence, in a particularly preferred embodiment, the precursors are derivatives of the estrogen substances, wherein the hydrogen atom of at least one of the hydroxyl groups in said formula has been substituted by $-\text{CO}-R$, wherein R is a hydrocarbon radical comprising from 1-25 carbon atoms. Preferably R is hydrogen, or an alkyl, alkenyl or aryl radical comprising from 1-20 carbon atoms.

The method according to the present invention may suitably be used to treat mammals such as cattle, pets and particularly humans. The method may be used to treat both females and males, be it that it is most beneficial when used in females. The method may be applied advantageously in premenopausal, perimenopausal and postmenopausal females.

The present method is particularly effective when the administration is continued for a prolonged period of time. Usually, the method comprises the uninterrupted administration of the estrogenic component during a period of at least 5 days. Preferably the uninterrupted administration is continued for at least 30 days, more preferably for at least 90 days.

5 The present method may suitably employ enteral or parenteral administration of the estrogenic component. The term "parenteral administration" as used in here encompasses transdermal, intravenous, intranasal, intravaginal, pulmonary, buccal, subcutaneous, intramuscular and intra-uterine administration. The term "enteral administration" includes oral as well as rectal administration.

10 Preferably the mode of administration is selected from the group consisting of oral, transdermal, intravenous, intranasal, intravaginal, pulmonary, rectal, buccal, subcutaneous, intramuscular or intra-uterine administration. More preferably the mode of administration is selected from the group consisting of oral, transdermal, intravenous, subcutaneous, intranasal, pulmonary and vaginal administration. In a particularly preferred embodiment the present
15 method employs oral, transdermal, intranasal or subcutaneous administration. Even more preferably the present method employs oral or transdermal administration.

Oral, intravenous, subcutaneous, intramuscular, intranasal, rectal, buccal and pulmonary administration are ideally suited for (at least) once daily administration. Transdermal and intravaginal administration are advantageously applied at frequencies
20 between once a day and once a month. Intra-uterine administrations is advantageously operated at administration frequencies between once weekly and once monthly. Subcutaneous and intramuscular administration may also suitably be done in the form of depot injections at intervals of 1 week to 6 months, preferably at intervals of 4 weeks to 3 months.

For reasons of convenience, the present method preferably utilises administration
25 intervals of 1 day, 1 week or 1 month. Regimens that employ once daily oral, subcutaneous, intravenous or intranasal administration, once weekly transdermal or once monthly intravaginal or subcutaneous administration are particularly preferred.

Irrespective of the mode of administration, the estrogenic component is preferably administered in an amount effective to achieve a blood serum concentration of at least 1
30 nanogram per litre, more preferably of at least 10 nanogram per litre, most preferably at least 100 nanogram per litre. Generally the resulting blood serum concentration of the estrogenic component will not exceed 100 μg per litre, preferably it will not exceed 50 μg per litre, more preferably it will not exceed 25 μg per litre.

In accordance with the present method the estrogenic component is usually administered in an amount of less than 1 mg per kg of bodyweight per day, preferably of less than 0.4 mg per kg of bodyweight per day. In order to achieve a significant impact from the administration of the estrogenic component, it is advisable to administer in an amount of at least 1 μ g per kg of bodyweight per day. Preferably, the administered amount is at least 5 μ g per kg of bodyweight per day.

Oral administration of the active component is preferably done in an amount of less than 400 μ g per kg of bodyweight per day, preferably of less than 200 μ g per kg of bodyweight per day. In order to achieve a significant impact from the administration of the active component, it is advisable to orally administer in an amount of at least 2 μ g per kg of bodyweight per day. Preferably, the orally administered amount is at least 5 μ g per kg of bodyweight per day. In the present method, particularly when used in humans, the estrogenic component is usually administered in an average dosage of at least 0.05 mg per day, preferably of at least 0.1 mg per day. The maximum dosage is normally kept below 40 mg per day, preferably below 20 mg per day.

The present method of treatment comprises administering to a mammal in need of such a therapy an effective amount of the estrogenic component. The amount needed to be effective will differ from individual to individual and are determined by factors such as the individual's gender, body weight, route of administration and the efficacy of the particular estrogenic component used.

In the present method, particularly when used in humans, the estrogenic component is usually administered orally in an average dosage of between 0.01 and 20 mg per day, preferably of between 0.05 and 10 mg per day. Similarly, the parenteral dosage preferably is at least 0.05, preferably at least 0.1 mg per day. The average maximum parenteral dosage is normally kept below 40 mg per day, preferably below 20 mg per day.

In a particularly preferred embodiment of the invention the method employs oral administration of the active estrogenic component. The term oral administration as used in here also encompasses oral gavage administration. The inventors have found that, despite its low potency, estetrol and related estrogenic substances may advantageously be administered orally. Although the inventors do not wish to be bound by theory, it is believed that the efficacy of orally administered estetrol-like substances results from the combination of special pharmacokinetic (ADME) and pharmacodynamic properties of these substances.

The inventors have discovered that the oral bioavailability of estetrol-like substances is exceptionally high and that their *in vivo* half-life is considerably longer than that of

commonly used biogenic estrogens. Thus, even though estetrol and estetrol-like substances have relatively low estrogenic potency, they may effectively be administered orally because the oral dosages required to achieve the desired effect are similar to those already used for e.g. 17 β -estradiol.

5 Another important advantage of oral administration of estetrol and estetrol-like substances resides in the fact that the hepatic effects of these substances are deemed to be minimal since they are hardly metabolised during the so called "first pass". The first-pass effect of drugs given orally refers to the process of drug degradation by the liver during a drug's transition from initial ingestion to circulation in the blood stream. After resorption from
10 the intestinal lumen, orally applied active ingredients enter the organism via the liver. This fact is of specific importance for estrogenic agents as the liver is a target organ for estrogens; oral intake of estrogens results in strong estrogenic effects in the liver. Therapeutically equivalent doses of commonly used biogenic estrogens, when applied orally, result in clear responses of hepatic parameters, such as increase of SHBG, CBG and angiotensinogen. These
15 hepatic effects of estrogens are also observed when equine estrogen formulations (so-called conjugated estrogens) are used.

The present method may suitably be used in the (prophylactic) treatment of various estrogen-suppressed tumours, including colorectal tumours and prostate tumours. In the case of treatment or prevention of prostate cancer, the present estrogenic component is suitably
20 administered in an amount effective to inhibit the endogenous production of androgens. The present method is most advantageously employed in the prevention or treatment of colorectal tumours, more preferably in the prevention or treatment of colon tumours.

In a particularly preferred embodiment of the present invention, the method is used to treat a mammal that suffers or has suffered from benign or malign tumours, particularly
25 colorectal tumours. The risk of an estrogen-stimulated cancer is deemed to be particularly high in mammals who have already developed tumours (including adenoma), even if these tumours have been surgically removed or otherwise eliminated. Consequently, the advantages of the present method are particularly pronounced in the treatment of such mammals as treatment with common estrogens would present a significant hazard. The present method is
30 most advantageously employed in the therapeutic treatment of a mammal suffering from estrogen-suppressed tumours.

In a preferred embodiment, the present method comprises the co-administration of a progestogen in an effective amount to suppress endogenous estrogen production. The co-administration of progestogen offers the additional advantage that progestogens are known to

inhibit the proliferative effect of estrogens on the endometrium. Although the present estrogenic components, unlike common estrogens, do not appear to have a pronounced proliferative effect on the endometrium, the co-administration of progestogen may be advisable to rule out any potential risks.

5 Examples of progestogens which may suitably be used in accordance with the present invention include: progesterone, levonorgestrel, norgestimate, norethisterone, dydrogesterone, drospirenone, 3-beta-hydroxydesogestrel, 3-keto desogestrel (=etonogestrel), 17-deacetyl
10 norgestimate, 19-norprogesterone, acetoxypregnenolone, allylestrenol, anagestone, chlormadinone, cyproterone, demegestone, desogestrel, dienogest, dihydrogesterone, dimethisterone, ethisterone, ethynodiol diacetate, flurogestone acetate, gastrinon, gestodene, gestrinone, hydroxymethylprogesterone, hydroxyprogesterone, lynestrenol (=lynoestrenol), medrogestone, medroxyprogesterone, megestrol, melengestrol, nomegestrol, norethindrone (=norethisterone), norethynodrel, norgestrel (includes d-norgestrel and dl-norgestrel), norgestrienone, normethisterone, progesterone, quingestanol, (17alpha)-17-hydroxy-11-
15 methylene-19-norpregna-4,15-diene-20-yn-3-one, tibolone, trimegestone, algestone acetophenide, nestorone, promegestone, 17-hydroxyprogesterone esters, 19-nor-17hydroxyprogesterone, 17alpha-ethinyl-testosterone, 17alpha-ethinyl-19-nor-testosterone, d-17beta-acetoxy-13beta-ethyl-17alpha-ethinyl-gon-4-en-3-one oxime and precursors of these compounds that are capable of liberating these progestogens *in vivo* when used in the present method.
20 Preferably the progestogen used in the present method is selected from the group consisting of progesterone, desogestrel, etonogestrel, gestodene, dienogest, levonorgestrel, norgestimate, norethisterone, drospirenone, trimegestone, dydrogesterone, precursors of these progestogens and mixtures thereof.

The invention is further illustrated by the following examples:

25

EXAMPLES

Example 1

30 A study is performed to examine the effect of estetrol on 1,2 dimethylhydrazine (DMH)-induced colon cancer in ovariectomized female rats.

DMH-induced cancer development is chosen as a model of "estrogen-suppressed tumours", because it mimics the human situation of colon carcinomas closely:

1) tumours grow orthotopically and can be classified as adenocarcinoma with the same histological tests,

2) the route of metastatic formation is the same (tumors grow relatively slowly and progress from adenoma to cancer) and

- 5 3) 17 β -estradiol confers protection in the model, as has been previously published (Greene et al., 1987, J. Surgical Research, 43, 476-487; Madara et al., 1983, Am. J. Pathology, 110, 230-235; Smirnoff et al., 1999, Oncology Research, 11, 255-264).

10 One week prior to induction of colon carcinomas, fifty sexually mature female Sprague-Dawley rats, weighing between 150 and 200 gram (Harlan, The Netherlands), are surgically castrated via removal of the ovaries. During the experiment, rats are maintained in separate plastic cages at 12 hour light/dark cycles and allowed free access to food (Purina chow) and water.

15 Animals are randomly allocated to one of five groups, each consisting of 10 rats, which, starting three days after ovariectomy and ending at autopsy, receive placebo or estetrol treatment during the experiment as follows:

- *Group 1* animals receiving placebo oral treatment with 3.0 ml/kg/day vehicle (20% wt/vol solution of hydroxypropyl-beta-cyclodextrin in water);
- *Group 2* animals receiving estetrol orally at a single daily dose of 0.1 mg/kg;
- *Group 3* animals receiving estetrol orally at a single daily dose of 0.3 mg/kg;
- 20 • *Group 4* animals receiving estetrol orally at a single daily dose of 1.0 mg/kg;
- *Group 5* animals receiving estetrol orally at a single daily dose of 3.0 mg/kg.

25 Starting one week after ovariectomy, animals from all groups are given subcutaneously, at weekly intervals, DMH injections (14.7 mg DMH dihydrochloride per 100 gram body weight) for 5 consecutive weeks, using a freshly prepared solution of DMH dihydrochloride in Hank's Balanced Salt Solution.

30 Fifteen weeks after the first DMH administration, the rats are sacrificed. At necropsy, each animal is incised and the colon is opened and examined closely for any grossly visible tumours before further processing for microscopic analysis. Criteria for determining malignancy are based on examination of histological features of the tumours and observing evidence for tumour invasion into submucosal layers. Only malignant tumours are included in the efficacy analysis.

In rats that have been treated with vehicle only, administration of DMH invariably results in the development of malignant colon tumours. In groups of animals treated with an

increasing dose range of estetrol as set forth, the number of malignant tumours declines as a function of increasing the daily oral estetrol dose from 0.1 to 3.0 mg/kg/day.

5 Example 2

Established competitive steroid binding assays were used to determine the relative binding affinity of estetrol (E4), as compared to 17 α -ethinylestradiol (EE) and 17 β -estradiol (E2), to human Estrogen Receptor (ER) α - and β -forms.

The method employed was adapted from the scientific literature and described in
10 detail by Osbourn et al. (1993, Biochemistry, 32, 6229-6236). Recombinant human ER α and ER β proteins were purified from transfected Sf9-cells. The *in vitro* assays involved the use of either ER α or ER β proteins and [3 H]E2, at a fixed concentration of 0.5 nM, as the labeled ligand. Recombinant human ER α or ER β proteins were dissolved in binding buffer (10 mM Tris-HCL, pH 7.5, 10% glycerol, 1 mM DTT, 1 mg/ml BSA) and duplicate aliquots were then
15 incubated with [3 H]E2 at a final concentration of 0.5 nM, together with a vehicle control (0.4% DMSO), or the same amount of vehicle containing increasing concentrations of unlabeled steroid ligands as competitors. After incubation for 2 h at 25 $^{\circ}$ C, the unbound ligands were removed and the amounts of [3 H]E2 bound to either ER α or ER β proteins were measured. The average amounts of [3 H]E2 bound to either ER α or ER β proteins at each
20 concentration of competitor were used to make inhibition curves. IC₅₀ values were subsequently determined by a non-linear, least squares regression analysis. Inhibition constants (K_i) were calculated using the equation of Cheng and Prusoff (Cheng et al., 1973, Biochem. Pharmacol., 22, 3099-3108), using the measured IC₅₀ of the tested compounds, the concentration of radioligand employed in the assay, and the historical values for the K_d of the
25 radioligand, which were established as 0.2 nM and 0.13 nM for ER α and ER β , respectively. Biochemical assay results for E4 are presented as the percent inhibition of specific binding in three separate experiments (Table 1). For comparison of binding affinities of E4, EE and E2 to human ER α and ER β proteins, experimentally observed K_i values are shown in Table 2. As compared to EE and E2, E4 demonstrates a unique binding profile with a strong
30 preference (400%) for binding to the ER α protein (Table 2). In contrast, K_i values for ER β protein are more pronounced for EE and E2 steroid ligands (Table 2).

Table 1: Percent inhibition of specific binding to ER α and ER β proteins using E4 as unlabeled steroid ligand and 0.5 nM [3H] as labeled competitor. Results of three separate experiments are shown.

E4 final concentration	Percent inhibition of specific binding in					
	ER α steroid binding assay			ER β steroid binding assay		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1 μ M	98	nd	nd	87	90	95
0.3 μ M	92	94	101	74	74	77
0.1 μ M	83	85	86	56	54	50
0.03 μ M	64	66	63	19	25	30
10 nM	43	32	28	nd	nd	nd
3 nM	26	17	11	nd	nd	nd

nd: not determined

- 5 **Table 2:** Experimentally determined inhibition constants (K_i) for estetrol (E4), 17 α -ethinylestradiol (EE) and 17 β -estradiol (E2), to human ER α and ER β proteins. Relative preference for binding to ER α protein is also shown.

Steroid ligands	K_i ER α (nM)	K_i ER β (nM)	Relative ER α /ER β preference(%)
EE	0.23	0.025	11
E2	0.21	0.015	7
E4	4.9	19	400

10

Example 3

In order to further assess the anti-tumour efficacy of the estrogenic substances of the present invention, estetrol was tested in the 7, 12-dimethyl-benz(a)anthracene (DMBA)-induced tumour model in rats. This model, originally developed by Huggins et al., 1961 (Nature, 19, 204-207), has been widely used and is a generally accepted model with predictive value for anti-tumour agents in humans. The growth of the DMBA-induced tumours in rats represents an example of "estrogen-stimulated cancers" and is dependent on endogenously produced estradiol or exogenously administered estrogens and prolactin (Sylvester et al., 1982, Cancer Research, 42, 4943-4947). Ovariectomy (Hollingsworth et al., 1998, Breast Cancer Research and Treatment, 47, 63-70), androgens (Dauvois et al., 1989, Breast Cancer

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Treatment, 14, 299-306), tamoxifen (Hollingsworth et al., 1998, Breast Cancer Research and Treatment, 47, 63-70), progestogens (Kelly et al. 1979, Eur. J. Cancer, 15, 1243-1251; Russo et al., 1987, Lab. Invest. 57, 112-137) and GnRH analogues (Hollingsworth et al., 1998, Breast Cancer Research and Treatment, 47, 63-70) all have been shown to be effective anti-tumour treatments in the DMBA model.

Eighty-four female Sprague-Dawley rats (Harlan, The Netherlands) were group housed, maintained in a 12-hr light/dark environment, and fed a Soya Free Diet (SDS England) and water ad libitum. Animals were weighed on a weekly basis. One week prior to induction of mammary carcinoma, 12 animals (aged 43 days) were surgically castrated via removal of the ovaries. At the age of 50 days, all animals were administered a single oral dose of 16 mg DMBA to induce tumour development. Animals were subsequently allocated to one of seven groups (n=12), receiving placebo or treatment as follows:

- *Group 1* animals received placebo oral treatment with 3.0 ml/kg/day vehicle (20% wt/vol solution of hydroxypropyl-beta-cyclodextrin in water);
- *Group 2* surgically castrated animals received placebo treatment with 3.0 ml/kg/day vehicle;
- *Group 3* animals received the anti-estrogen tamoxifen given orally at a single daily dose of 3 mg/kg;
- *Group 4* animals received ethinylestradiol (EE) orally at a single daily dose of 0.025 mg/kg;
- *Group 5* animals received ethinylestradiol (EE) orally at a single daily dose of 0.125 mg/kg;
- *Group 6* animals received estetrol (E4) orally at a single daily dose of 0.5 mg/kg; and
- *Group 7* animals received estetrol (E4) orally at a single daily dose of 2.5 mg/kg.

The doses of EE and E4 were based on data from previous studies, showing equipotency of 0.025 mg/kg/day EE and 0.5 mg/kg/day E4 in agonistic models of preventing bone resorption, prevention of hot flushing and vaginal cornification. Similarly, the doses of 0.125 mg/kg/day EE and 2.5 mg/kg/day E4 showed equipotency in *in vivo* estrogenicity in preventing bone resorption, prevention of hot flushing and vaginal cornification.

During the treatment period of 8 weeks, the emergence of palpable tumours and number of tumours were determined weekly. At 8 weeks, at necropsy, final measurements were taken. The number of tumours at necropsy are depicted in figure 1. As is clearly demonstrated by the absence of tumours in the ovariectomized animals (group 2), development of DMBA-induced mammary tumours is estrogen-dependent. As expected, also

tamoxifen showed anti-tumour properties by inhibiting the development of mammary tumours in this model. Surprisingly, and in contrast to the effect seen with the 0.125 mg/kg/day dose of EE, E4 at an equipotent agonistic dose of 2.5 mg/kg/day markedly suppressed mammary tumour development. Furthermore, this particular dose of E4 was as effective as tamoxifen in preventing growth of DMBA-induced tumours.

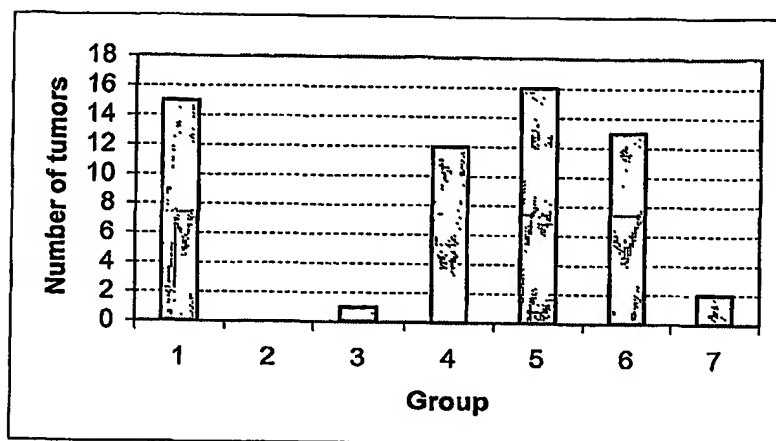


Figure 1. Number of tumours per treatment group (n=12).

Group 1 oral treatment with 3.0 ml/kg/day vehicle;

10 *Group 2* surgically castrated animals receiving placebo treatment with 3.0 ml/kg/day vehicle;

Group 3 tamoxifen 3 mg/kg/day orally;

Group 4 ethinylestradiol (EE) 0.025 mg/kg/day orally;

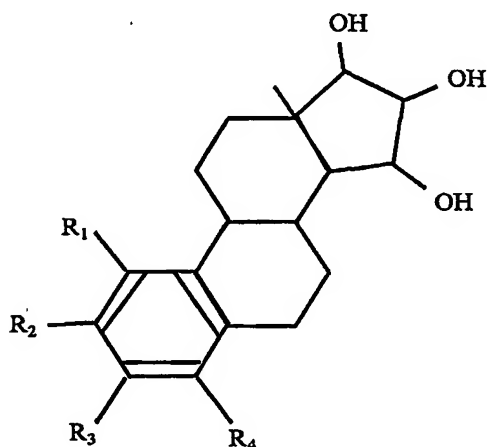
Group 5 EE 0.125 mg/kg/day orally;

Group 6 estetrol (E4) 0.5 mg/kg/day orally;

15 *Group 7* E4 2.5 mg/kg/day orally.

CLAIMS

1. Use of an estrogenic component selected from the group consisting of:
substances represented by the following formula



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in which formula R_1 , R_2 , R_3 , R_4 independently are a hydrogen atom, a hydroxyl group
or an alkoxy group with 1-5 carbon atoms;

precursors capable of liberating a substance according to the aforementioned formula when
used in the present method; and

- 10 mixtures of one or more of the aforementioned substances and/or precursors;
in the manufacture of a pharmaceutical composition for use in a method of treating or
preventing estrogen-suppressed tumours in a mammal, said method comprising the
administration of a therapeutically effective amount of the estrogenic component to said
mammal.

15

2. Use according to claim 1, wherein no more than 3 of R_1 , R_2 , R_3 , R_4 are hydrogen
atoms;
3. Use according to claim 1 or 2, wherein R_3 represents a hydroxyl group or an alkoxy
20 group.
4. Use according to any one of claims 1-3, wherein at least 3 of the groups R_1 , R_2 , R_3 and
 R_4 represent hydrogen atoms.

5. Use according to any one of claims 1-4, wherein the method comprises the uninterrupted administration of the estrogenic component during a period of at least 5 days, preferably of at least 30 days.

5

6. Use according to any one of claims 1-5, wherein the method comprises oral, transdermal, intravenous or subcutaneous administration of the estrogenic component.

7. Use according to claim 6, wherein the method comprises oral administration.

10

8. Use according to any one of claims 1-7, wherein the estrogenic component is administered in an amount of at least 1 μg per kg of bodyweight per day, preferably of at least 5 μg per kg of bodyweight per day.

15 9. Use according to any one of claims 1-8, wherein the estrogen-suppressed tumours are selected from the group consisting of colorectal tumours or prostate tumours.

10. Use according to any one of claims 1-9, wherein the mammal suffers or has suffered from benign or malign tumours, particularly colorectal tumours.

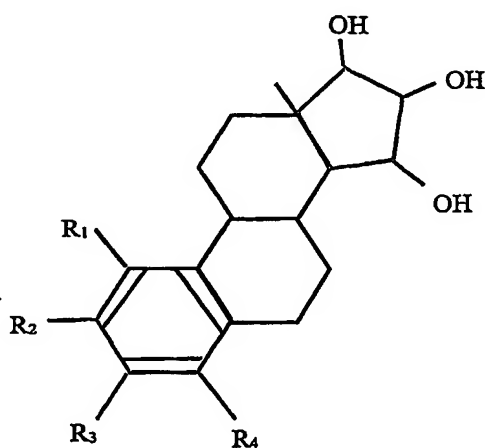
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11. Use according to any one of claims 1-10, wherein the mammal is a female, particularly a human female.

25 12. Use according to any one of claims 1-11, wherein the method comprises co-administration of a progestogen.

ABSTRACT

The present invention relates to a method of treating or preventing estrogen-suppressed tumours in a mammal, said method comprising the administration of a therapeutically effective amount of an estrogenic component to said mammal, wherein the estrogenic component is selected from the group consisting of:
5 substances represented by the following formula



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in which formula R₁, R₂, R₃, R₄ independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms; precursors capable of liberating a substance according to the aforementioned formula when used in the present method; and mixtures of one or more of
10 the aforementioned substances and/or precursors.

The estrogenic component according to the invention is particularly useful in the treatment or prevention of colorectal and prostate cancer and, unlike commonly used estrogens, does not simultaneously enhance the risk of estrogen-stimulated cancers such as
15 breast cancer.

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